ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF HALO AND THERMO TOLERANT BACTERIA FROM COSTAL RIDGE OF MEDITERRANEAN IN EGYPT

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Extremophiles, the microbes dwelling in unusual habitates, can potentially serve in a variety of industrial applications. As a result of adaptation to extreme environments, extremophiles have evolved unique properties that make them of biotechnological and commercial significance (Margesin and Schinner 2001; Dodia *et al.*, 2006). Extremophiles include halophiles, alkali philes, acidophiles, thermophiles and haloalkaliphiles. Hypersaline lakes are mostly populated with halophilic neutrophilic organisms. The halophiles were described (Horikoshi, 1999) as a subgroup that requires high salinity (up to 33% W/V NaCl). The organisms living in such extreme environment possess special adaptation strategies that make them interesting not only for fundamental research but also towards exploration for applications. In the present study, one natural saline habitat along the Mediterranean cost in north Egypt was selected for the isolation followed by the identification and molecular characterization of halotolerant and thermophilic bacteria.

MATERIALS AND METHODS

Sampling

Soil samples were collected from the hyper saline environments, Costal ridge, Mallahat Maryut, Bourg El-Arab and El-Amiriya that are located along the Mediterranean in the North of Egypt. Soil samples were taken from depths of about 15 cm. Soil samples were serially diluted from the stock concentration 10 g soil/100 ml sterile saline solution, then 0.1 ml were inoculated on a medium containing (g/Liter): Peptone 5 g, Yeast extract 3 g, Beef extract 1 g and Sodium chloride at different concentrations (0.5 M, 1 M, 2 M, 3 M, 4 M, 4.5 M and 5.2 M) and incubated at different temperature degrees, 30, 37, 55, 75 and 80°C for 2-14 days. Isolated bacteria were counted as (colony forming unit) CFU/g soil (Gebreel, 1999).

Morphology and cytology

Morphological characteristics of the colony, as appearance, elevation and production of pigments were examined on

nutrient agar (Peptone 5 g, Yeast extract 3 g, Beef extract 1 g and Sodium chloride 5 g, adjust pH to 7 and agar was added 15 g/L) (Ventosa *et al*., 1982). Motility was observed under microscope using the hanging drop technique; log phase of bacterial growth was examined by a high-power dry objective reduced illumination. Motility was confirmed by stabbing the organism in semisolid medium and after 7 days of incubation, diffusion of growth was recorded as positive results. Cell morphology was examined after Gram reaction (Dussault, 1955). The purified colonies were subjected to spore stain for microscopic examination; endospore shape and position were characteristic of *Bacillus* species. Formation of insecticidal crystal proteins (ICP) were the gold standard, since it directly identifies *Bacillus thuringiensis* from closely related Bacilli as *B. cereus* and *B. anthracis*.

Biochemical and physiological properties

Physiological and biochemical tests were performed as described in Bergey's manual of systematic bacteriology (Claus and Berkeley, 1986). Bacterial isolates were tested for catalase production when it was reacted with 3% hydrogen peroxide. Oxygen was released from catalase producer microorganisms (Cowen and Steel, 1974). For acetyl-methylcarbinol production, methyl red and Vogues-proskauer (MR-VP) tests were done. A drop of methyl red solution was added to a 7 day-old bacterial culture. Bright red color refers to positive MR. 0.6 ml of 5% α-naphthol solution and 0.2 ml of 40% potassium hydroxide (Berret's reagent) was added onto 1 ml of that bacterial culture. The development of pink crimson color indicates a positive VP. The ability to reduce nitrate was examined as described by (Cowen and Steel, 1974). To test for sugar fermentation, 0.1% of glucose or other source of carbohydrates were added to a medium containing peptone water (Peptone 2 g, D-Glucose 1 g, Sodium Chloride 5 g) and the indicator Bromothymol blue with derhum tube were used (Hugh and Leifson, 1953).

Total protein analysis

Total cellular protein of bacterial isolates were grown on LB media (Trypton 10 gm, yeast 5 gm, sodium chloride 5gm) at 30°C over night compared to that grown on 30% NaCl. SDS-PAGE was carried out as described by (Laemmli, 1970). The bacterial cells were collected by centrifugation and treated with sample buffer composed of (50 mM Tris-HCl, pH 6.8, 2% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.0025% (w/v) bromophenol blue) and boiled at 100°C for 5 min. Samples were applied to a 10% polyacrylamid gels and run in mini protein Biorad cell. Protein bands on gels were visualized with Coomassie brilliant blue R-250. Total cellular proteins of sporulated bacterial cells from *Bt* isolates were also prepared. Those bacterial cells were grown on T3 medium (tryptone 3 gm, tryptose 2 gm, yeast extract 1.5 gm, sodium phosphate

buffer 50 mM, Mn Cl_2 0.005 gm, in 1L dH_2O) for 72 h in shaking incubator at 200 rpm and 30°C. Samples were applied to a 10% polyacrylamid gels as mentioned.

Polymerase chain reaction (PCR) and oligonucleotide primers

Two pairs of specific primers for *cry*1C were used, CJ10 & CJ11, 5' AAAGATCTGGAACACCTTT3' & 5'CAAACTCTAAATCCTTTCAC3' that amplifies a 130 bp fragment of DNA and CJ1-1 & CJ1-2, 5' TGTAGAAGAGGAAGTCTATCCA3' & 5' TATCGGTTTCTGGGAAGTA3' that amplifies a 284 bp fragment of DNA (Ceron *et al*., 1995) and 273 bp in case of *cry* 1Ac. Another two pair of specific primers to *cry*1C gene were also used to test the presence of *cry*1C in the *B. thuringiensis* isolates. The two primers IAF and IAR (Regev *et al*., 1996). IAF, 5' ACGGAGGATCCATATGGAGGAAAA TAATCAAAATC3' and IAR, 5'CTCTTGGATCCTAACGGGTATAAG CTTTTAATTTC3', that give 2.2 k bp PCR product. The reaction conditions were performed according (Regev *et al*., 1996), where the PCR mixture was in a total volume of 25 µl contained 1 µg of total DNA, 50 pmol of each primer, 0.2 mM deoxy nucleoside triphosphates (dNTP), 2.5 µl of the Taq polymerase enzyme, 2.5 µl of 10 X enzyme buffer and 2.5 µl Mg Cl_2. The amplification reaction was carried out using 35 cycles of 94°C denaturation (45 sec), 48°C annealing (45 sec) and 72°C extensions (120 sec) and then a 7- min termination at 72°C. The same reaction conditions were used with the second pair of primers IAF and ICR, 5'TTATTCCTCCATAAGGAGTAATTC C3' (Ibrahim, 2001), that give 3.7 kbp PCR product. The *gln* B specific pair of primers that define the nitrogen regulatory gene were used, *nif*D up & do specific pair of primers were also used to detect both the two genes *gln* B and *nif* D in all the four isolates (S7, 8, 9 and 10). *Gln* B up 5'GCCATCATTAAGCCGTTCAA3' and *gln* B do 5'AAGATCTTGCCGTCGCCGAT3' and *nif*D up 5'ATCATCGGTGACTACAAC3' & *nif*D do 5'ATCCATGTCGCGGCGAA3'. The reaction conditions were as described by (Potrich *et al*., 2001), 250 bp PCR products are amplified by *gln*B pair of primers and 710 bp by *nif*D primers.

RESULTS AND DISCUSSION

Halotolerant and thermotolerant bacterial isolates were counted on plates containing nutrient agar medium that contained different concentrations of sodium chloride (0.5-24%). Mesophilic and halotolerant bacteria were incubated at 30°C while the thermophilic and halotolerant were incubated at 55°C and the thermotolerant isolates were incubated at 75°C in presence of 10% sodium chloride. The results showed that there were great variations in the number of the viable bacteria per gram-soil in different locations. The highest population of bacteria was obtained in Costal ridge

which reached 3.0X105 CFU / g soil at 24% sodium chloride concentration and incubation temperature 30°C and 50°C (Fig. 1), and in Burg El-Arab which reached 4.5x104 CFU / g soil in presence of 10% NaCl concentration and incubation temperature 75°C (Fig. 2). Ten isolates were selected for further studies which were the most halotolerant (able to grow at 30% NaCl) and thermotolerant (able to grow at 75°C incubation temperature) ones. The isolates did not display much diversity as regards to their colony characteristic (Table 1). However, the cell morphology and arrangement did vary significantly among these isolates from the same location. The gram positive character was abundant among these isolates and their colony pigmentation and texture had limited variations. All the isolates were characterized with endospore formation and the position of those endospores varied from central to terminal (Table 1). Six isolates were able to grow over a wide range of salt (up to 30% NaCl "5.2 M"), the other 4 isolates could not grow over this high range but 10% NaCl was optimum to its growth and they were characterized with the ability to grow on high temperature up to 75°C. The biochemical and the physiological tests (Table 2) revealed that all the isolates belong to the Bacillus species which showed positive gram reaction and ellipsoidal endospore production. The isolates were facultatively aerobes, they produced catalase, reduced nitrate to nitrite and produced acid from D-glucose during fermentation. Six isolates produced acetylmethylcarbinol, did not produce indol, utilized citrate and they could hydrolyze gelatin, starch and urea. Mature spores were central in position for 6 isolates while they were terminal in the other 4 isolates (Table 1). The 6 isolates (S1, 2, 3, 4, 5 and 6) produced characteristic crystals for the insecticidal toxin protein in *B. thuringiensis* and the microscopic examination clearly showed that crystals (Fig. 4A). The other 4 isolates were identified as *paenibacillus polymyxa* according the biochemical tests of the systematic classification of Bergey's. Identification of our isolates was agreed with (Seija Elo *et al*., 2001).

Protein analysis

Total protein analysis of sporulated cells from the *B. thuringiensis* isolates revealed the presence of major bands belonging to the insecticidal crystal protein (ICPs) (Fig. 3), S1 showed main band near the 116 kDa, S2 showed major band at ~135 kDa that typically related to the lepidopteran Cry protein and also showed other main band at ~ 68 kDa that typically related to coleopteran Cry protein. S3 also showed expressed major protein band at ~ 135 kDa, S4 expressed main protein bands at 116, 97 and 66 kDa. S5 was characterized with the presence of main band under 116 kDa and S6 showed very interesting major band slightly over that was expressed by S3 "above 135 kDa". It was very interesting that the main bands of that *B. thuringiensis* isolates appeared at the same concentration when the isolates were grown on LB liquid media overnight. On the protein synthesis

level, as in (Mojica *et al*., 1997) when bacterial cells of *Halomonas elangata* were grown at different salinities, there were long-term response, also they showed induction of protein synthesis after a shift of high-salt conditions. Salt adaptation could be indicated by a gradually higher accumulation of certain proteins in cells growing under gradually increasing salinities. Mojica *et al.* (1997) found that among such proteins were 39, 24 and 15.5 kDa not detected at low salt concentration (0.3% NaCl) and all of these proteins related to the long-term response to high salt concentrations. Our data revealed high concentration of two protein bands at ~ 60 kDa and 30 kDa in isolate S2 (Fig. 4B), two bands at~ 95 kDa and ~ 28 kDa in the isolate S3 (Fig. 4A) and disappearance of a band above 116 KDa. In S1 high concentration of a protein band at ~ 100 kDa. In S4 at ~ 65 kDa, ~ 37 kDa and 28 kDa (Fig. 4C). Thus our results agreed with Mojica *et al*. (1997) who found that the proteins that were synthesized at high salt concentrations were on long-term response, up to 7 days on shaker. Proteins homologous to those previously described as having a role in osmoregulation in bacteria such *E. coli* as mentioned by (Csonka and Hanson, 1991) are expected to be found in this study.

Protein analysis for four *B. polymyxa* bacterial isolates were detected and compared with 3 strains identified as *B. polymyxa*. The protein banding pattern of our isolates was similar to that of reference strains in some protein bands, like the band that found less than 37 kDa and the protein bands that found at molecular masses between 116 and 70 kDa. Our isolates characterized with main bands; S7 showed characteristic major band under 66 kDa, S8 and S10 showed major different band above that band of S7 (Fig. 5), S9 also showed a characteristic band; further studies on these isolates may lead to identification of protein related to the high temperature which these isolates were able to grow on 75°C. Microscopic examination of bacterial cells of our isolates that were grown on T3 medium for 72 h, revealed the presence of the insecticidal crystal protein (ICP) in the six *B. thuringiensis* isolates and the shape of crystals were different from isolate to other while it was typically bipyramidal as that characterized Cry 1C crystal (Ibrahim and Omar, 2005) in S2, S3 and S6. In the remaining isolates the crystal shapes were different (Fig. 6A). On the other hand, the 4 *B. polymyxa* isolates showed similar shape of spores (Fig. 6B).

PCR analysis

B. thuringiensis crystal producing isolates have been isolated that are insecticidal against Lepidopterans, Dipterans and Coleopterans. Nucleotide sequences reported for *B. thuringiensis* crystal protein genes represent 14 distinct genes (Hofte and Whiteley, 1989). Cry 1A crystal protein genes exhibiting insecticidal activity against lepidopterans share extensive DNA sequence homology (Carozzi *et al*., 1991). Thus PCR technology and primers specific for *B. thur-*

ingiensis delta-endotoxin genes to develop a rapid screen of new *B. thuringiensis* isolates that can predict their insecticidal activities were used. In our work 6 primers were chosen to give characteristic product profiles from the genes encoding major class (lepidopterans) of *B. thuringiensis* crystal proteins. *Cry* 1C - specific primers CJ10 & CJ11 give a 130 bp product and CJ1-1 & CJ1-2 give a 284 bp product with *cry* 1C (Ceron *et al*., 1994 & 1995). IAF & IAR give 2.2 kb (Regev *et al*., 1996). Isolates S2, S3, S5 and S6 gave the expected products with the 1st and 2nd pair of primers (Fig. 7A) while S2 and S3 also gave the expected 2.2 kb product with the 3rd pair of primers but they didn't give the expected 3.7 kb product that represent the full length of the most published *cry* 1C with the primer pair IAF & ICR and this is a preliminary indication that *cry* 1C of S2 and S3 may differ from other *cry* 1C (Fig. 7B). On the other hand a set of primers was designed from previously published sequences of *gln* B gene (the nitrogen regulatory gene) and *nif* D gene (Potrich *et al*., 2001) and used with other 4 isolates that supposed to be *B. polymyxa*. All the 4 isolates gave the 250 bp product with *gln* B specific primers (Fig. 7C) and the 710 bp product with the *nif* D specific primers (Fig. 7D). These results put these *B. polymyxa* isolates in the diazotrophic *paenibacillus polymyxa*.

In summary, in this work ten bacterial isolates dwell in extreme habitats (high salt, high temperature, and low oxygen) were isolated and selected from a huge number of isolates. Six of these isolates were *Bacillus thuringiensis* that tolerate high salt concentrations of NaCl up to 30% and temperature up to 55°C, most of these *Bt* isolates were characterized by the lepidopteran insecticidal crystal proteins. They differ in molecular masses and shape of crystals from the common Cry 1C. The presence of osmoregulatory proteins that were synthesized at high salt concentrations may convoy us to reveal that we isolated strong isolates of *Bt* and may be novel and could be used in biological control of insect pests. In this work also 4 isolates of *B. polymyxa* were isolated which were able to grow on high temperature 75°C and contained the nitrogen fixing genes *gln* B and *nif* D. These isolates seemed of great importance for the biofertilizers of crops cultivated in hot areas.

SUMMARY

Costal ridge of Mediterranean and the regions near by it, like Mallahat Maryut, Bourg El-Arab and El-Amiriya in the north of Egypt are suitable places for the isolation of halo- and thermo-tolerant microorganisms. This study presents isolation, identification and molecular characterization of halotolerant and thermophilic bacteria. Ten bacterial isolates were isolated using enrichment techniques, up to 30% (W/V) NaCl and 75°C incubation temperatures. Isolates were identified according to Bergey's Manual as *Bacillus* species, six isolates were identified as *Bacillus thuringiensis* and the other four isolates were *B. polymyxa.*

Protein profiling of the *Bt* isolates and the PCR detection of *cry* genes showed the presence of lepidopteran *cry*1C gene. On the protein synthesis level, the protein profiling of cells grown in 5.2 M NaCl showed or/ disappeared a set of high-salt related proteins. On the other hand, the other 4 isolates of *B. polymyxa* were able to grow at 75°C. Protein profiling and the PCR detection of *gln*B & *nif*D genes revealed the presence of these genes. Thus *B. polymyxa* isolates could be used to fix nitrogen on the crops cultivated in hot regions.

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Table (1): Colony, morphology and endospores characteristics of Halotolerant and Thermotolerant bacterial isolates from saline locations in North of Egypt.

Isolates	Colony Character					Vegetative cells morphology		Endospore characters		
	Form	Margin	Texture	Pigmentation	Elevation	Shape	Arrangement	Shape	Position	Crystals
1	Circular	Entire	Smooth & Shining	Orange	Convex	Rods	Long Chain	Ellipsoidal	Central	+Ve
2	Rhizoid	Rhizoidal	Smooth & Shining	Cream white	Convex	Rods	Long Chain	Ellipsoidal	Central	+Ve
3	Circular	Entire	Smooth & Shining	Pale orange	Convex	Rods	Long Chain	Ellipsoidal	Central	+Ve
4	irregular	Filamentous	Smooth & Shining	White	Convex	Rods	Long Chain	Ellipsoidal	Central	+Ve
5	Circular	Entire	Smooth & Shining	Yellowish white	Convex	Rods	Long Chain	Ellipsoidal	Central	+Ve
6	Circular	Entire	Smooth & Shining	Yellowish Cream	Convex	Rods	Long Chain	Ellipsoidal	Central	+Ve
7	Rhizoid	Rhizoidal	Smooth	White	Flate	Rods	Short Chain	Ellipsoidal	Terminal	-Ve
8	Circular	Entire	Smooth	White	Flate	Rods	Short Chain	Ellipsoidal	Terminal	-Ve
9	irregular	Filamentous	Smooth	White	Flate	Rods	Short Chain	Ellipsoidal	Terminal	-Ve
10	Rhizoidal	Rhizoidal	Smooth	White	Flate	Rods	Short Chain	Ellipsoidal	Terminal	-Ve

Table (2): Biochemical and physiological tests used for identification of the bacterial isolates.

Characteristics	Bacterial Isolates (S1, 2, 3, 4, 5 & 6)	Bacterial isolates (S7, 8, 9 & 10)
Gram Stain	+ve	+ve
Motility	+ve	+ve
Catalase test	+ve	+ve
Anaerobic growth	+ve	+ve
Vogas-Prokauer test	+ve	-ve
Formation of Indole	-ve	-ve
Acid from :		
D-glucose	+ve	+ve
L-Arabinose	-ve	+ve
D-Xylose	-ve	+ve
D-Mannitol	-ve	+ve
Gas from Glucose	-ve	+ve
Utilization of Citrate	+ve	+ve
Nitrate reduction test	+ve	+ve
Hydrolysis of Casein	-ve	+ve
Hydrolysis of gelatin	+ve	+ve
Hydrolysis of Starch	+ve	+ve
Hydrolysis of Urea	+ve	-ve

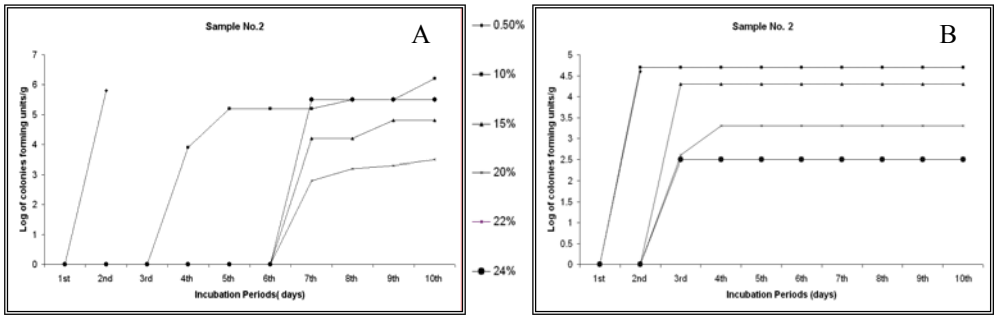
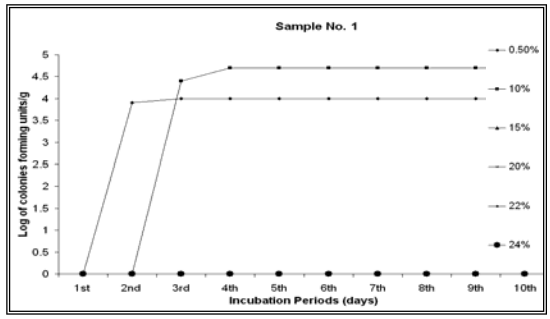


Fig. (1): Total bacterial count in soil sample collected from Costal ridge region after different incubation periods at 30°C (A) and 50°C (B) represented in colony forming units (cfu/g) in presence of different concentrations of NaCl in nutrient agar medium.

Fig. (2): Total bacterial count in soil sample collected from Burg El-Arab region after different incubation periods at 75°C, colony forming units (cfu/g) in presence of different concentrations of NaCl in nutrient agar medium.



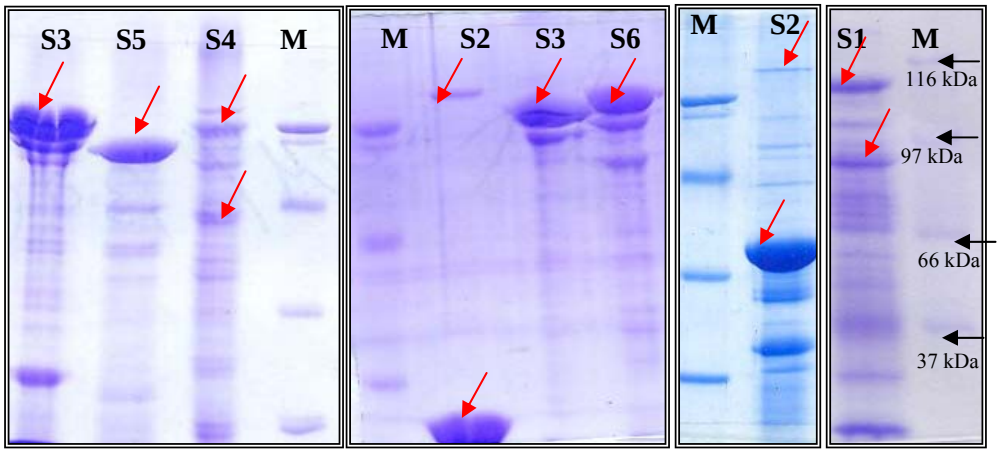


Fig. (3): SDS-PAGE analysis of total cellular proteins of sporulated bacterial cells of the 6 *B. thuringiensis* isolates. 25 µl of total cellular proteins were run on 10% polyacrylamide gels. The gels were stained with Coomassie blue. M: is a protein marker of 116, 97, 66, 37 and 28 kDa and was used with all the gels presented here. The name of each isolate is written on its lane. The red arrows refer to the major bands of the insecticidal crystal protein (ICP) that characterized each isolate.

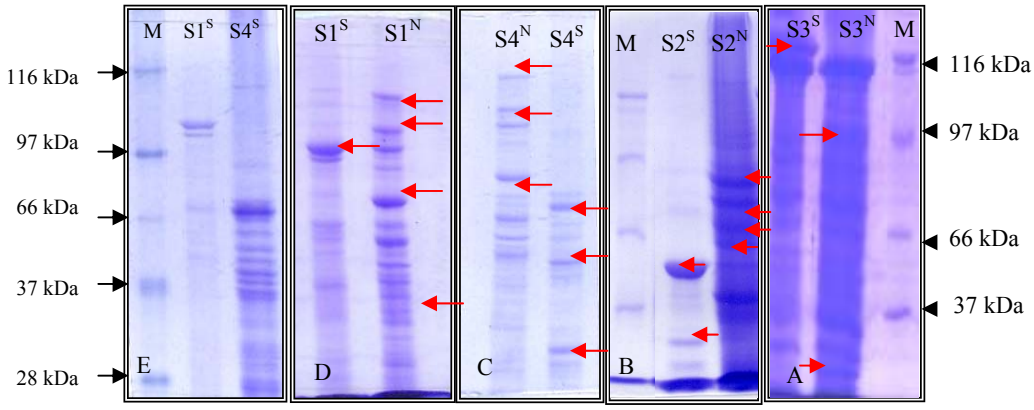


Fig. (4): Effects of variations of salinity on protein synthesis in *B. thuringiensis* isolates. Sporulated bacterial cells of 4 *Bt* isolates (S1, S2, S3 and S4) that were grown under high salt concentrations (30% NaCl) compared to that grown under normal salt concentrations (0.1 NaCl). 25 µl of total cellular proteins were run on 10% poly acrylamide gels. The gels were stained with Coomassie blue. M: is a protein marker of 116, 97, 66, 37 and 28 KDa and was used with all the gels presented here (A, B and E). The name of each isolate is written on its lane (^{N} means normal & ^{S} means high salt conc.). The red arrows refer to protein bands that were found in case of normal salt (A:$S3^{N}$, B: $S2^{N}$, C: $S4^{N}$, D: $S1^{N}$) and become faint or disappeared in case of high salt concentration or refer to unique bands that appeared in case of high salt concentrations (A: $S3^{S}$, B: $S2^{S}$, C: $S4^{S}$, D: $S1^{S}$). E: contain protein marker "M" beside $S1^{S}$ and $S4^{S}$.

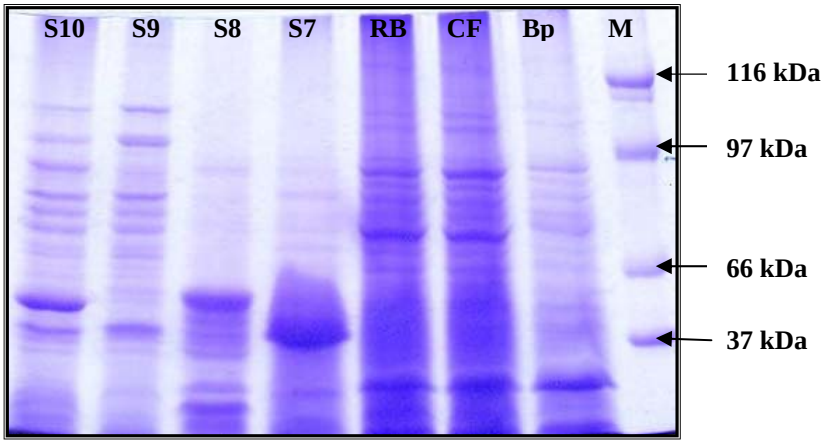


Fig. (5): SDS-PAGE analysis of total cellular proteins of bacterial cells of 4 isolates S7, S8, S9 and S10 which are expected to be *B. polymyxa* compared to the protein pattern of 3 strains of bacteria *B. polymyxa*; RB, CF and Bp. The name of each isolate or strain is written on its lane. M: protein marker and its molecular kilo-daltons are written beside it.

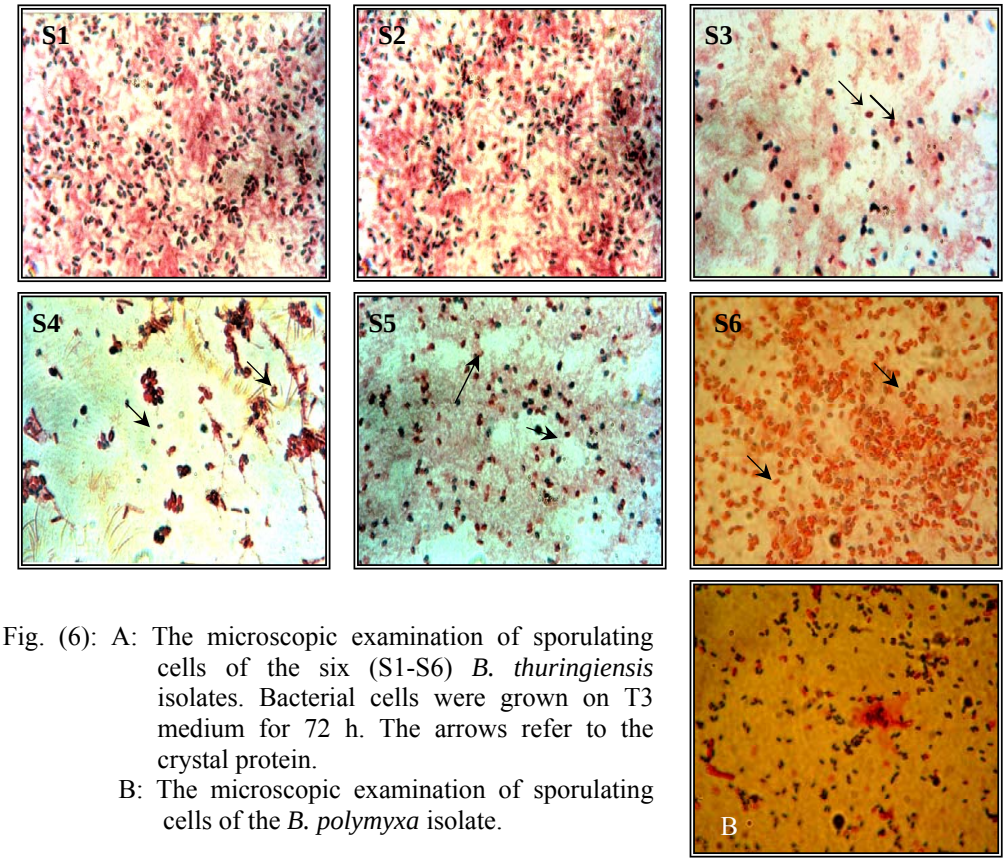


Fig. (6): A: The microscopic examination of sporulating cells of the six (S1-S6) *B. thuringiensis* isolates. Bacterial cells were grown on T3 medium for 72 h. The arrows refer to the crystal protein.
B: The microscopic examination of sporulating cells of the *B. polymyxa* isolate.

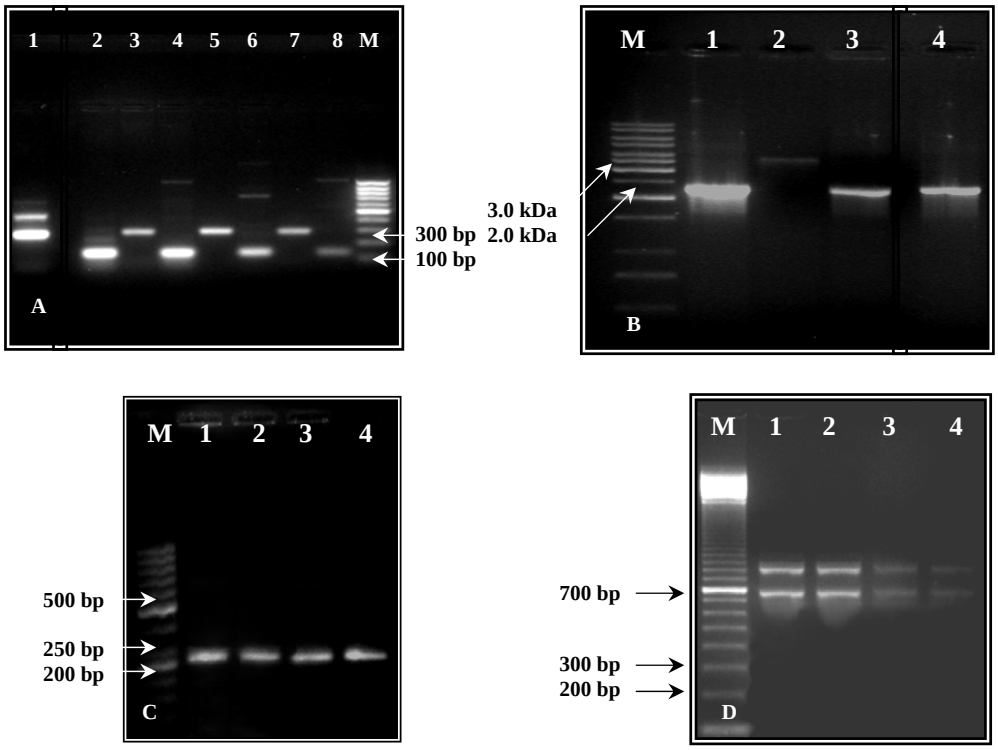


Fig. (7): Agarose gel electrophoresis for amplified PCR products from DNA of bacterial isolates.

A: PCR detection of lepidopteran *cry* gene, the primers CJ10 & CJ11 (give 130 bp PCR product with *cry* 1C) and CJ1-1 & CJ1-2 (give 284 bp PCR product with *cry* 1C and 272 bp with *cry* 1Ac) were used. Lane1: S6 with CJ1-1 & CJ1-2 (give 284 bp PCR product). Lane2: S6 with CJ10 & CJ11 (130 bp PCR product). Lane3: S5 with CJ1-1 & CJ1-2. Lane4: S5 with CJ10 & CJ11. Lane5: S3 with CJ1-1 & CJ1-2. Lane 6: S3 with CJ10 & CJ11. Lane7: S2 with CJ1-1 & CJ1-2. Lane8: S2 with CJ10 & CJ11. M: 100 bp DNA molecular marker.

B: The primer sets IAF & IAR (give 2.2 kb with *cry* 1C) and IAF & ICR (give 3.7 kb with *cry* 1C) were used.
M: 1 kb ladder DNA molecular marker. Lane1: Positive control with IAF&IAR (2.2 kb PCR product). Lane2: Positive control with IAF&ICR (3.7 kb PCR product). Lane3: S2 with IAF&IAR. Lane4: S3 with IAF&IAR.

C: The primers set *gln*B up & *gln*B do that give 250 bp PCR product with *gln*B gene were used. M: 50 bp DNA molecular marker. Lane1: S7 with glnB up&do (250 bp product). Lanes 2, 3 and 4 are S8, S9 and S10 with *gln*B up&do.

D: The primer set *nif*D up&do that give 710 bp PCR product with *nif*D gene were used. M: 100 bp ladder DNA molecular marker. Lanes 1, 2, 3 and 4 are S7, S8, S9 and S10 with *nif*D up&do.